JOURNAL OF BACTERIOLOGY, Oct. 1983, p. 463–465
0021-9193/83/100463-03$02.00/0

Vol. 156, No. 1

Mutation of the *N*-Acetylmuramyl-L-Alanine Amidase Gene of *Escherichia coli* K-12

SHIGEO TOMIOKA, TOSHIO NIKAIDO, TOKICHI MIYAKAWA,† AND MICHIO MATSUHASHI*
Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo, 113 Japan

Received 3 January 1983/Accepted 5 July 1983

Mutants of *Escherichia coli* with very low *N*-acetylmuramyl-L-alanine amidase activity were isolated. The gene *amiA* responsible for most of this enzyme activity was mapped at 51 min on the *E. coli* chromosome, with the most plausible gene order assumed to be *amiA pts(H* or *I) purC*. The mutant phenotype was recessive and physiologically undiscernible.

N-Acetylmuramyl(MurNAc)-L-alanine amidase, which hydrolyzes the amide linkage between glycan and peptide in peptidoglycan, is one of the major cell wall lytic enzymes in gram-positive bacteria and also is responsible for postfissional separation of cells (2, 5, 10). On the other hand, the enzyme with a similar name in gram-negative bacteria does not hydrolyze cell wall peptidoglycan but only splits the MurNAc-L-alanine linkage of smaller molecules that do not contain *N*-acetylglucosamine (13). Therefore, functions of the enzyme in gram-negative bacteria are unknown. Recently, we isolated a mutant of *Escherichia coli* that lacks almost all the activity of this enzyme. The mutant cell was viable and showed no distinct phenotypical changes. The gene *amiA*, which is responsible for the enzyme mutation, was mapped, and a double mutant involving the *envA* mutation, which is supposed to function in regulating the level of the amidase activity, was constructed.

Characterization of the MurNAc-L-alanine amidase activity that became defective by the *amiA* mutation. Enzyme assay was carried out with MurNAc-tripeptide (L-Ala-D-Glu-*meso*-[1,7-^{14}C]diaminopemilic acid (A_2pm) as substrate. The reaction mixture for the amidase assay contained, in a final volume of 30 μl, 50 mM Tris-hydrochloride buffer (pH 7.5), 67 pmol of labeled MurNAc-tripeptide (specific activity, 50 Ci/mol) and crude cell extract (supernatant of 6,000 × *g* [10 min] centrifugation) containing 30 to 70 μg of protein. The mixture was incubated at 30°C for various times, and the reaction was terminated by boiling the mixture for 90 s. The radioactive product and substrate were separated by paper electrophoresis in 0.1 M formic acid (11 V/cm, 2 h), and the radioactivities on the paper were counted in a liquid scintillation spectrometer in toluene–2,5-diphenyloxazole–1,4-bis-(5-phenyloxazolyl)benzene (1 liter:4 g:100 mg). The amidase reaction obeyed first-order kinetics until about 40% of the substrate was degraded. The amidase activity was calculated from the amounts of disappearance of substrate and appearance of the product after calibration on the basis of a first-order reaction. Evidence that the amidase activity in the mutant was not reduced by the presence of an inhibitor was obtained in an experiment with a mixture of sonic cell extracts of the parent and the mutant; the amidase activity in the *amiA* strain (parent) was neither inhibited nor stimulated by the presence of twice as much (as protein) of the *amiA* mutant cell extract.

A single product was detected after incubation with cell extracts of *amiA*$^+$ strains. The product tripeptide possessed one molecule of alanine with a free N-terminal which was not present in the substrate. The free N-terminal of alanine in the product was identified and determined quantitatively by converting it to the 2,4-dinitrophenol (DNP)-tripeptide, followed by acid hydrolysis and separation of DNP-alanine by thin-layer chromatography from mono-DNP-A_2pm, which was obtained from both the substrate and product. From 1 μmol each of the substrate and the product, 0.05 and 1.09 μmol of DNP-alanine and 1.05 and 1.09 μmol of mono-DNP-A_2pm, respectively, were obtained. These values are corrected for the recoveries of DNP-alanine (0.48) and DNP-A_2pm (0.47).

Isolation and characterization of mutants. Mutants with reduced MurNAc-L-alanine amidase activity were isolated from the collection of temperature-sensitive mutants of *E. coli* K-12 strain JE1011 (F$^-$ *thr leu trp his thyA thi ara lac gal xyl mtl rpsL azi*) (7). The cells were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1) and therefore also carried multiple other mutations on their chromosomes (8). Two mu-

† Present address: Department of Fermentation Industry, University of Hiroshima, Hiroshima, Japan.

tant strains, JST752 and JST738, were isolated from 50 temperature-sensitive mutants by survey for a defect in amidase activity in the cell sonicate. Cells were grown in 7 ml of modified Lennox broth containing, per liter, 10 g of tryptone (Difco Laboratories), 5 g of yeast extract (Difco), 5 g of NaCl, 1 g of glucose, and 20 mg of thymine, adjusted to pH 7.0 with NaOH to the late log phase (absorbance at 660 nm, about 0.8), and sonicate was prepared (12). Strain JST752 had only 5% (0.032 nmol per mg of protein per h) of the activity of the parent (0.68 nmol per mg of protein per h), whereas activity of strain JST738 was about 17% that of the parental activity. Most of the amidase activity in the crude extracts of the parent strain JE1011 was found in the supernatant obtained at 100,000 × *g* (60 min), with only a small amount found in the precipitated membrane fraction. The amidase activities in both fractions of mutant JST752 were reduced significantly. No appreciable changes in the activities of other lytic enzymes, such as penicillin-insensitive DD-endopeptidase (12), DD-alanine carboxypeptidases (11), LD-alanine carboxypeptidase (4), or anhydro-muramidase (3) activities could be detected in the mutants. In the following genetic experiments, only mutant JST752 was used.

Map position of *amiA* mutation and dominance test. The amidase mutation in strain JST752 (referred to as *amiA*) was mapped by sexual cross with Hfr strains and then by transduction with phage P1. As the *amiA* mutation did not cause any detectable change in the phenotypes, recombinants were identified by enzyme assay on each recombinant.

Short-term mating with the Hfr strain KL-16 (HfrH *thi-1 rel-1*) or PK191 (Hfr [O *supD*] *thi-1* DE5 [*proB lac*] *supE44*) indicated that the *amiA* gene is located on the region of the chromosome between *thyA* and *his*. Transduction experiments indicated that the *amiA* gene was cotransducible with *purC* (52.8 min) with 4 to 5% frequency and with *pts*(*H* or *I*) (51.7 min) with 70% frequency. Thus, it was concluded that the *amiA* gene was close to the *pts* gene on the chromosome.

Three point tests with phage P1 were carried out, and the results showed that *amiA* is not located between *purC* and *pts* (Expt 1, Table 1). The possibility that *amiA* was located outside *purC* was also excluded (Expt 2, Table 1). The remaining possibility is that *amiA* is outside *pts*. This possibility was supported by the results in Expt 3 (Table 1). Thus, the most plausible gene order is *amiA pts purC*.

Amidase activity measured in two isogenic pairs of strains involving the *amiA*$^+$ and *amiA* genes is shown in Table 2 (Expt 1).

A dominance test with a meroheterodiploid obtained from the *amiA* strain by mating with F'198 (point of origin KL198) that carries the chromosomal part from *supN* to *nadB* (6) and curing by treatment of the *amiA*(F') strain with acridine orange indicated that the *amiA* mutation was recessive to the wild type (Expt 2, Table 1). Thus, the *amiA* gene could be the structural gene of the amidase or the gene that positively regulates amidase production.

Phenotype of the *amiA envA* double mutant. Normark (9) reported that mutation in the *envA* gene (2 min) caused formation of chains of cells and a decrease in the MurNAc-L-alanine amidase activity when the cells were grown in a rich medium (14). We prepared coupled pairs of isogenic strains involving *envA*$^+$ and *envA* genes from each strain of *amiA*$^+$ and *amiA*. As shown in Expt 3, Table 2, the *envA* strains that were selected by short-chain cell shape and supersensitivities to ampicillin, rifampin, and gentian violet (9) had a level of amidase activity similar to that of the isogenic *envA*$^+$ cells. The amidase

TABLE 1. Three point tests by phage P1 transduction

Expt no.	Genetic marker[a]			Strains and selections			Total no.
	purC	*pts*	*amiA*	1[b]	2[c]	3[d]	
1	1	1	1	10	6	15	31
	1	1	0	1	1	2	4
2	1	1	1		6	4	10
	0	1	1		4	1	5
3	1	0	1	0[e]			0
	1	0	0	29			29

[a] Genetic markers are expressed as 1 (donor type) and 0 (recipient type). M9 broth supplemented with 20 mg of thymine per liter, 4 g of glucose per liter, and 50 mg of required nutrients per liter were used for Pur$^+$ selection. For culturing Pts$^-$ cells, 0.2% glucose-6-phosphate was used instead of glucose. Pts$^+$ cells were selected on a plate of M9 containing 4 g of glucose per liter, and the isolated transductants were tested for sensitivity to fosfomycin (100 mg/liter).

[b] Donor, strain JST752 (*purC*$^+$ *pts*$^+$ *amiA*); recipient, strain TMH677 (F$^-$ *trp-45 his-68 tyrA2 purC50 thi-1 lacY1 gal-6 xyl-7 mtl-2 malA1 rpsL125 tonA2 pts*(*H* or *I*) *tsx-70 supE44* λ^r λ^-, spontaneous Pts$^-$ mutant of H677 [CGSC4900]); first selection, Pur$^+$; second selection, Pts$^+$ or Pts$^-$.

[c] Donor, strain JST752; recipient, strain TMH677; first selection, Pts$^+$; second selection, PurC$^+$ or PurC$^-$.

[d] Donor, strain H677; recipient, strain JST7522 (spontaneous Pts$^-$ mutant of JST752); first selection, Pts$^+$; second selection, Pur$^+$ or Pur$^-$.

[e] One recombinant with an intermediary amidase level was obtained among 30 recombinants after the second selection for Pts$^-$.

TABLE 2. MurNAc-L-alanine amidase activity in wild-type and mutant-type cells of *E. coli*

Expt no.	Strain[a]	Genotype[b]	Amidase activity[c]	Cell shape
1	TMH6771	*amiA pts*$^+$ *purC*	0.04	Normal rod
	TMH6772	*amiA*$^+$ *pts*$^+$ *purC*	0.58	Normal rod
	TMH6773	*amiA pts*$^+$ *purC*$^+$	0.04	Normal rod
	TMH6774	*amiA*$^+$ *pts*$^+$ *purC*$^+$	0.58	Normal rod
2	JST7521	*amiA pts*	0.04	Normal rod
	JST7521(F198)	*amiA pts/amiA*$^+$ *pts*$^+$	0.72	Normal rod
	JST7521*	*amiA pts*	0.04	Normal rod
3	JAG11	*amiA*$^+$ *envA*$^+$	0.90	Normal rod
	JAG12	*amiA*$^+$ *envA*	0.80	Short chain
	JAG21	*amiA envA*$^+$	0.07	Normal rod
	JAG22	*amiA envA*	0.06	Short chain

[a] Strains TMH6771, TMH6772, TMH6773, and TMH6774 are isogenic transductants (donor, JST752; recipient, TMH677; selection, Pts$^-$ for TMH6771 and TMH6772, Pur$^+$ for TMH6773 and TMH6774). JST7521 (genotype, same as JST752 but *pts*(*H* or *I*) *recA thy*$^+$) was obtained from JST752 by spontaneous *pts*(*H* or *I*) mutation and mating with KL16-99 (HfrH *thi-1 rel19 recA1 drm39*). JST7521* was obtained by treating JST7521(F198) with 30 mg of acridine orange per liter. Strains JAG11 and JAG12 were derived from strain JAG2260 (F$^-$ *amiA*$^+$ *thr leu trp his argG thyA thi ara lac gal xyl mtl rpsL azi*) by transduction with phage P1 lysate from strain D22 (F$^-$ *envA proA trp his ampA rpsL lacY*, obtained from S. Normark). From Leu$^+$ recombinants *envA*$^+$ and *envA* strains were selected by their cell shape and supersensitivities to rifampin, gentian violet, and aminobenzylpenicillin. Strains JAG21 and JAG22 were derived from strain JAG2250 (*amiA* strain isogenic to JAG2260) by transduction as described above.

[b] Genotypes closely located to *amiA*, *amiA*, and *envA* are listed.

[c] For amidase assay, see text.

activity of the *amiA envA* double mutants was also similar to that of the isogenic *amiA envA*$^+$ strain. Thus, we could not so far detect a decrease in activity in *envA* cells. Moreover, no formation of longer chains of the *amiA envA* double mutants was observed.

We are indebted to B. J. Bachmann for supplying mutant strains from the Coli Genetic Stock Center, Yale University, New Haven, Conn.

This work was partially supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan.

LITERATURE CITED

1. **Adelberg, E. A., M. Mandel, and G. C. C. Chen.** 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. **Fein, J. E., and H. J. Rogers.** 1976. Autolytic enzyme-deficient mutants of *Bacillus subtilis* 168. J. Bacteriol. **127**:1427–1442.
3. **Höltje, J. V., D. Mirelman, N. Sharon, and U. Schwarz.** 1975. Novel type of murein transglycosylase in *Escherichia coli*. J. Bacteriol. **124**:1067–1076.
4. **Izaki, K., and J. L. Strominger.** 1968. Biosynthesis of the peptidoglycan of bacterial cell walls XIV. Purification and properties of two D-alanine carboxypeptidases from *Escherichia coli*. J. Biol. Chem. **243**:3193–3201.
5. **Koyama, T., M. Yamada, and M. Matsuhashi.** 1977. Formation of regular packets of *Staphylococcus aureus* cells. J. Bacteriol. **129**:1518–1523.
6. **Low, K. B.** 1972. *Escherichia coli* K-12 F-prime factors, old and new. Bacteriol. Rev. **36**:587–607.
7. **Miyakawa, T., M. Matsuzawa, M. Matsuhashi, and Y. Sugino.** 1972. Cell wall peptidoglycan mutants of *Escherichia coli* K-12: existence of two clusters of genes, *mra* and *mrb*, for cell wall peptidoglycan biosynthesis. J. Bacteriol. **112**:950–958.
8. **Nikaido, T., S. Tomioka, and M. Matsuhashi.** 1979. Mutant of *Escherichia coli* tolerant to the lysis induced by cephalexin. Agric. Biol. Chem. **43**:2639–2640.
9. **Normark, S.** 1970. Genetics of a chain-forming mutant of *Escherichia coli*. Genet. Res. **16**:63–78.
10. **Pooley, H. M., G. D. Shockman, M. L. Higgins, and J. Porres-Juan.** 1972. Some properties of two autolytic-defective mutants of *Streptococcus faecalis* ATCC 9790. J. Bacteriol. **109**:423–431.
11. **Tamura, T., Y. Imae, and J. L. Strominger.** 1976. Purification to homogeneity and properties of two D-alanine carboxypeptidases 1 from *Escherichia coli*. J. Biol. Chem. **251**:414–423.
12. **Tomioka, S., and M. Matsuhashi.** 1978. Purification of penicillin-insensitive DD-endopeptidase, a new cell wall peptidoglycan-hydrolyzing enzyme in *Escherichia coli*, and its inhibition by deoxyribonucleic acids. Biochem. Biophys. Res. Commun. **84**:978–984.
13. **Van Heijenoort, J., C. Parquet, B. Flouret, and Y. Heijenoort.** 1975. Envelope-bound N-acetylmuramyl-L-alanine amidase of *Escherichia coli* K-12. Eur. J. Biochem. **58**:611–619.
14. **Wolf-Watz, H., and S. Normark.** 1976. Evidence for a role of *N*-acetylmuramyl-L-alanine amidase in septum separation in *Escherichia coli*. J. Bacteriol. **128**:580–586.